Post-translational Analysis of *Arabidopsis thaliana* Proteins in Response to Cyclic Guanosine Monophosphate Treatment

Thesis by
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In Partial Fulfillment of the Requirements
For the Degree of
Master of Science

King Abdullah University of Science and Technology
Thuwal, Kingdom of Saudi Arabia
December 2011

EXAMINATION COMMITTEE

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ABSTRACT

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The introduction of mass spectrometry techniques to the field of biology has made possible the exploration of the proteome as a whole system as opposed to prior techniques, such as anti-body based assays or yeast two-hybrid studies, which were strictly limited to the study of a few proteins at a time. This practice has allowed for a systems biology approach of exploring the proteome, with the possibility of viewing entire pathways over increments of time. In this study, the effect of treating *Arabidopsis thaliana* suspension culture cells with 3',5'-cyclic guanosine monophosphate (cGMP), which is a native second messenger, was examined. Samples were collected at four time points and proteins were extracted and enriched for both oxidation and phosphorylation before analysis via mass spectrometry. Preliminary results suggest a tendency towards an increased number of phosphorylated proteins as a result of cGMP treatment. The data also showed a sharp increase in methionine oxidation in response to the treatment, occurring within the first ten minutes. This finding suggests that cGMP may utilize methionine oxidation as a mechanism of signal transduction. As such, this study corroborates a growing body of evidence supporting the inclusion of methionine oxidation in intracellular signaling pathways.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank Prof. Chris Gehring for his generous support and supervision. His concern for me as an aspiring young man was as enriching as his concern for the results of my research, and I am grateful for having had the chance to work under him and with him.

I would also like to thank Dr. Ludivine Thomas and Dr. Claudius Marondedze for their supervision, aid and support, as well as the opportunity to join their project after it had already been begun. The two of them provided me with all of the protein samples and aided me in preparing most of them all of the way up to where I began data analysis. Additionally, they have been a constant resource during the writing of this thesis and during analysis of the data. The techniques and understanding I have gained from the project are significant, and it could not have been accomplished without the aid of my dedicated post-docs and supervisors.

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LIST OF ABBREVIATIONS

ACN: Acetonitrile

cGMP: cyclic Guanosine Monophosphate

CREB: cAMP-Responsive Element Binding

DHB: 2,5-dihyroxybenzoic Acid

MS: Mass Spectrometry, Mass Spectrometer

MSR: Methionine Sulfoxide Reductase

P3DB: Plant Protein Phosphorylation Database

PMCA: Plasma Membrane Calcium-ATPase

ROS: Reactive Oxygen Species

SCX: Strong Cation Exchange

TCA: Trichloroacetic Acid

TFA: Trifluoroacetic Acid

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1. INTRODUCTION

An improved understanding of cellular signaling is an important focus of research due to its importance in almost every biological process. Whether a scientist is interested in studying drought tolerance, finding a cure for cancer or in exploring the implications of genetics on behavior, cell signaling is at the core of the issue. In all of these cases, a proper understanding of the subject is one that must originate at the level of a single cell. It is thus important to be able to explore the signal(s) the cell is sensing and the response(s) triggered. The development of techniques for accomplishing this task continues to be a very active field of research [1-3].

Cell signaling has most commonly been studied at the transcriptome level [4] since the base-pairing property of DNA and RNA makes detection of these sequences more straightforward than detection of proteins. For example, a 2006 study was searching for down-stream targets of the second messenger 3',5'-cyclic guanosine monophosphate (cGMP) and used microarray technology to determine that genes regulating specific ion channels are upregulated in response to cGMP treatment [5]. However, this method is inherently flawed, as, for the most part, it is the proteins, not the RNA templates from which they come, that actually accomplish tasks within the cell. Though RNA templates provide a proxy for the amount of their related protein, it has been shown that measurements of the transcriptome are not always a good representation of the proteome [6]. Exploration of the proteome has posed a significant challenge in the quest to better illuminate cellular signaling,

because most methods of observing protein expression have traditionally been time-consuming and have only allowed for the study of a few proteins at a time. However, highly sensitive mass spectrometry (MS) equipment, highly accessible databases of genomic data, and computational hardware and software capable of using mass spectrometry data to examine entire proteomes, currently provide a valuable tool in analyzing protein samples in order to determine which proteins are actually expressed. This technology has developed to the point where it is now possible even to investigate post-translational modifications such as phosphorylation or oxidation of amino acid residues [1, 3]. This allows for the analysis of signaling pathways in their entirety, and provides snapshots of the proteins expressed in a cell, as well as their modifications, in response to signaling or other factors.

With this technology in mind, it is possible to explore plant signaling in response to specific molecules, focusing on post-translational modifications. This study focuses specifically on cGMP, which is a second messenger involved in many signaling pathways. Many plant hormones and stress conditions induce responses that include an increase in cellular cGMP levels, but the effects of cGMP seem to include post-translational modifications in addition to changes in transcription. Therefore, exploration of cGMP's role in signaling is important because it is commonly present and yet its downstream targets are not well characterized [5]. *Arabidopsis thaliana* is a suitable experimental organism for this study because of its rapid growth cycle and well characterized genome and transcriptome [7].

2. MATERIALS AND METHODS

2.1 MATERIALS

All chemicals and materials used in the study were obtained from Sigma-Aldrich (Taufkirchen, Germany) except where indicated.

2.2 METHODS

2.2.1 Culturing of *Arabidopsis thaliana* cells *

(* indicates procedure completed prior to arrival in lab)

Cells used for the experiment were grown in a liquid cell suspension culture. This culture was subcultured into fresh media every 3-4 weeks. The media used for the culture was Gamborg's medium, which consists of 3.2g/L Gamborg's B5 medium including vitamins (Sigma G5893), 30g/L of sucrose, 0.5g/L MES, 0.5 mL/L of 2,4-D stock, and 50 μL/L of kinetin stock. The hormone stocks each consisted of the appropriate hormone in a 1 mg ml^{-1} concentration. The media was adjusted to pH 5.7 with KOH. 250ml flasks were filled with 50ml of Gamborg's medium and inoculated with callus, and then placed on a shaker at 120 rpm, 23°C in a 12 h light/12 h dark cycle.

2.2.2 8-Br-cGMP treatment of callus cells *

After two weeks of culturing, the callus cells were subjected to sterile treatment with cell-permeable cGMP analogue. 20 μl of 8-Br-cGMP solution (10μg/μl) was injected into three flasks containing callus cells resuspended in

medium, so that the final concentration of cGMP analogue was 10 μM, while cells in additional flask were not treated with the reagent. The control cells were harvested using sterile filtration system (Millipore), and cGMP analog treated cells from three flasks were harvested in the same way at different time points – 10, 30 and 60 minutes. Subsequently, cells were transferred to separate 50 ml Falcon tubes and frozen in liquid nitrogen. The tubes were kept in -80°C and thawed prior to protein extraction.

2.2.3 Protein Extraction *

Protein extraction from samples containing cells harvested at four different time points was conducted simultaneously, in separate Falcon tubes. A 1.5g weight of callus cells, kept at -80°C, was homogenized with mortar and pestle in liquid nitrogen and subjected to protein precipitation in ten volumes of cold 10% TCA in acetone, while the rest of callus cells were kept at -80°C as a backup. After vortexing, the extract was incubated overnight at -20°C. The pellet containing proteins was collected by centrifugation of the samples for 20 minutes at 4000 rpm at 4°C, and the supernatant was discarded. The pellet was washed four times, each time with at least 10 volumes of cold 80% acetone, vortexed and centrifuged in the same conditions. In order to remove the residual acetone, the pellet was air-dried for approximately 10 minutes, and subsequently resuspended in urea/thiourea/Chaps lysis buffer, containing inhibitors of phosphatases (Calbiochem) and proteases (Roche) in concentrations recommended by manufacturers - 1:5 and 1:10 dilutions, respectively. After overnight vortexing, the lysate was separated from the cell debris

by 10 minutes centrifugation 4000 rpm at 4°C followed by discarding of the pellet. Protein content in supernatants was quantified using Bradford method.

2.2.4 Protein quantitation *

Protein quantitation was performed using Quick Start Bradford Reagents (Bio-Rad) according to manufacturer's instruction. After warming the reagent to room temperature, 2 mg/ml BSA stock solution supplied in the kit was used to prepare 19 the standard curve. Double replicates of samples 1-6 were prepared (Table 1), ingredients of each were mixed well and incubated for five minutes. Similarly, double replicates of the investigated sample, in volume of 20 µl, was mixed with 1 ml of Bradford reagent and incubated for five minutes. Absorbance of each sample was read at the wavelength of 595 nm using Helios Epsilon UV-Vis spectrophotometer (Thermo Scientific), scaled to zero with probe 1.

2.2.5 Protein reduction/alkylation and digestion with trypsin *

Protein reduction was done by addition of appropriate volume of reduction stock solution (0.5 M DTT) to the protein extract, so the final concentration of DTT equal to 5 mM, and incubation of the mixture for 2 h at 37°C. After cooling down, the concentration of IOA in the extract was increased to 14 mM by addition of appropriate volume of alkylation stock solution (1.4 M IOA). 30-minute incubation of the mixture at room temperature in the dark enabled cysteine residues to become alkylated. Subsequent increase in DTT concentration to 10 mM and 15-minute incubation at room temperature in the dark quenched the unreacted IOA. Protein

extract was diluted in 10 ml of 50 mM TEAB buffer (pH 8.5) and subjected to overnight digestion with 100 µg of trypsin (Worthington) at 37°C. The reaction was terminated by acidification with TFA to the final concentration of 0.4% (vol/vol) and pH adjustment to the value below 2.0. After centrifugation of the digest at 4000 rpm for 10 minutes at 20°C the supernatant underwent desalting, and the pellet was discarded.

2.2.6 Tryptic peptides separation using Strong Cation Exchange (SCX) Chromatography

2.2.6.1 Standard preparation *

A mixture of BSA tryptic peptides was used as a standard. 80 µl of 50 mM TEAB buffer (pH 8.5) and 40 µl of 10 mg/ml BSA solution were added to 1 ml vial of trypsin (Worthington). The mixture was transferred to 2 ml micro-centrifuge (Eppendorf) tube, and additional 960 µl of 10 mg/ml BSA solution was added. After vortexing, 15 µl of ACN was added for reduction, and the mixture was vortexed again. Overnight digestion took place at 37°C. The solution of BSA tryptic peptides was aliquoted into 40 µl volumes, corresponding to single run on the SCX column, and kept in freezer at -20°C.

2.2.6.2 Desalting of tryptic peptides from Arabidopsis thaliana *

Peptide digest was desalted using Sep-Pak Vac tC18 desalting columns (Waters). The 500 mg cartridge was prepared according to the manufacturer's recommendations. Briefly, the column was conditioned with 9 ml of 100% ACN,

washed with 3 ml of 50% ACN and 0.5% HAcO and 9 ml of 0.1% TFA. After loading the sample in 0.4% TFA, the cartridge was washed with 9 ml of 0.1% TFA and with 900 µl of 0.5% HAcO to remove TFA. Elution was performed with 5 ml of 50% ACN and 0.5% HAcO, and the eluate was frozen in dry ice and underwent overnight freeze-drying.

2.2.6.3 Running the standard and samples on SCX column*

After 7-minute priming Dionex HPLC system with degassed buffers A, B, C and D, PolySulfoethyl A column was equilibrated with buffers A, B and A (each for 0.5 h) at the flow rate of 0.5 ml/min. 5-cm loop was flushed with 5 ml of buffer A, and 40 µl standard of BSA tryptic peptides was thawed, diluted in 3 ml of buffer A and injected into the loop. When peptides elution with buffer B was finished, the SCX column was equilibrated with buffer A. Desalted and freeze-dried sample of tryptic peptides, with protein concentration estimated for 5 mg, was diluted in 1 ml of buffer A and injected into the loop. The progress of peptide separation was monitored on chromatogram (absorbance signal at λ = 214 nm and λ = 280 nm versus retention time). Gradient elution program (Table 2) was run, first 5 min eluate was discarded and from this time on 30 1.5-ml fractions were manually collected in 1.5-ml LoBind tubes micro-centrifuge (Eppendorf) tubes for further analysis - 17 fractions corresponding to early peaks on chromatogram were desalted on 100 mg cartridges (Waters) and subsequently freeze-dried, while remaining 13 fractions were frozen and kept at -80°C.

2.2.6.4 Desalting of selected elution fractions from SCX column *

Fractions of elution that presumably corresponded to phosphopeptides underwent desalting process on 100 mg tC18 Sep-Pak Vac extraction cartridges (Waters). The cartridges were washed and conditioned with 3 ml of ACN and then with 3 ml of 50% ACN 0.5% HAcO. After equilibration with 3 ml of 0.1% TFA, samples in 0.1% TFA were loaded. Each cartridge was desalted with 3 ml of 0.1% TFA, and TFA was removed by washing with 250 µl of 0.5% HAcO. Elution was conducted using 1 ml of 50% ACN 0.5% HAcO, and eluates were collected in 1.5-ml LoBind micro-centrifuge (Eppendorf) tubes, frozen in dry ice, and after overnight freeze-drying they were kept at -80°C.

2.2.7 Phosphopeptide Enrichment

Enrichment of phosphopeptides was conducted using TiO_2 beads (GL Sciences, Torrance, USA). For 17 freeze-dried SCX fractions, 40 mg of TiO2 beads was suspended in 2 ml of 100% (v/v) ACN and spun down at 4000 g to collect the pellet, and the supernatant was kept on a shaker for a minimum of 5 min and subsequently spun at 4000 g and then supernatant was discarded. Beads were equilibrated in 400 μ l of washing buffer (80% (v/v) ACN and 2% TFA), vortexed, and centrifuged at 4000 g to remove supernatant. Then the pellet of beads was dissolved in 200 μ l of the loading buffer (1.3M DHB, 80% (v/v) ACN and 2% TFA), and freeze-dried SCX elution fractions were suspended in 50 μ l of the loading buffer, vortexed for at least 5 min and kept on a shaker. To each fraction, a volume of

10 μ l of TiO2 beads resuspended in the loading buffer was added. The mixture was vortexed, spun down and incubated on a shaker for 5 min. Subsequently, phosphopeptides conjugated with the beads were pelleted at 4000 g for15 sec at room temperature, 30 μ l of supernatant was discarded, the beads were transferred into 200 μ l C18 Proxeon tips, and minicolumns of TiO2 beads were formed by pushing the liquid through the tips with a syringe. The minicolumns were washed three times with 45 μ l of washing buffer, and phosphopeptides were eluted in a three-step elution firstly with 30 μ l of the elution buffer 1 (0.5% (v/v) ammonia solution), and then with 10 μ l of elution buffer 2 (30% ACN (v/v)). Eluates from all three stages were collected in the same 1.5-ml micro-centrifuge tubes for each fraction. After puncturing the lids, micro-centrifuge tubes containing the eluates were frozen in ice, dried overnight in a vacuum drier centrifuge and stored at -20° C until it was used for MS analysis.

2.2.8 Preparation of sample for analysis using LTQ-Orbitrap mass spectrometer

Trypsin digested peptide mixtures were resuspended in 0.1% (v/v) formic acid (FA) in 5% (v/v) ACN, and analyzed on an LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) coupled with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark) for nano-LC-MS/MS analyses. A volume of 5 µL of peptide mixtures was injected onto a 50 mm long × 0.3 mm Magic C18AQ (Michrom) column. A spray voltage of 1,500 V was applied. The mobile phases

consisted of 0.1% FA and 5% ACN (A) and 0.1% FA and 90% ACN (B). A three step gradient of 0-40% B in 20 min, then 40-90% B in 5 min and finally 90% B for 20 min with a flow of 500 nL min-1 over 45 min was applied for peptide elution. The MS scan range was m/z 350 to 1,600. The top 10 precursor ions were selected in the MS scan by Orbitrap with resolution r = 60,000 for fragmentation in the linear ion trap using collision induced dissociation. The normalized collision-induced dissociation was set to 35.0.

All spectra were submitted to a local MASCOT (Matrix Science, London, UK) server and searched against Arabidopsis thaliana in the TAIR (release 10), with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.6 Da, and strict trypsin specificity allowing up to one missed cleavages, carbamidomethyl modification on cysteine residues as fixed modification and oxidation of methionine residues and phosphorylation of serine, threonine and tyrosine residues as variable modifications. Proteins were considered positive if the Mascot score was over the 95% confidence limit corresponding to a score ≤26 for plant. Protein spectra were then processed with ProteinProphet for validation.

2.2.9 Data Analysis

A database of *Arabidopsis thaliana* phosphoproteins was downloaded from P3DB (http://www.p3db.org/, September 2011) for determining proteins that were previously identified as phosphoproteins [8].

3. PHOSPHORYLATION

3.1 INTRODUCTION

Phosphorylation is a form of posttranslational modification that results in activation and deactivation of proteins as part of intracellular signaling. Proteins are generally phosphorylated by kinases and dephosphorylated by phosphatases. The means of effecting a change in activity of the phosphorylated protein is via a change in conformation associated with the addition of a highly negative charged and hydrophilic phosphate group. Such changes often serve to open up active sites enabling the protein to accomplish its action. The well-known phosphorylation cascade is an example of how phosphorylation of a few kinases can lead to an exponentially increasing number of phosphorylated proteins, which accomplish various signaling tasks such as induced calcium influx [9]. Processes such as this can fundamentally alter the behavior of a cell. Thus, it is highly important to study these pathways to gain a better understanding of which proteins are involved and how they interact to effect a signal. It is of particular interest to examine which proteins react to specific signaling molecules, as it would be expected that specific downstream signaling molecules may cause phosphorylation or signal transduction among only a subset of proteins, thus allowing for less complexity while attempting to map interactions. Linking the phosphoproteome to the phenotypic response of the plant may also hasten elucidation of the phenotypic effects of complicated signaling pathways. In this preliminary research, the focus was primarily on

determining which proteins are phosphorylated in response to cGMP treatment in order to understand its potential effects and the signaling pathway involved.

In this preliminary study, results for one replicate of each time point were obtained, and the other two replicates are yet to be analyzed. This will then pave the way for a confident statistical interpretation of the data. The focus of this section was to identify phosphorylated proteins in response to cGMP treatment.

3.2 RESULTS

Therefore, enriched phosphopeptides were identified using the Orbitrap Velos MS and then verified using the Plant Protein Phosphorylation Database (P3DB) [8], a database of known curated plant phosphorylated proteins. However, some proteins not reported in the database were identified as phosphorylated proteins by mass spectrometry and the data consistently showed evidence of phosphorylation among them. Though the ratio in most time points strongly favored proteins that were found in the database, the identification of phosphorylated proteins not present in the database, if accurate, indicates that there were unique protein phosphorylation events that have not previously been documented (**Figure 1**). Additionally, the data for confirmed phosphoproteins show a trend of increasing phosphorylation in response to the cGMP treatment. The data related to unconfirmed phosphoproteins also exhibits this trend if the 30 min data point is dismissed as an experimental anomaly.

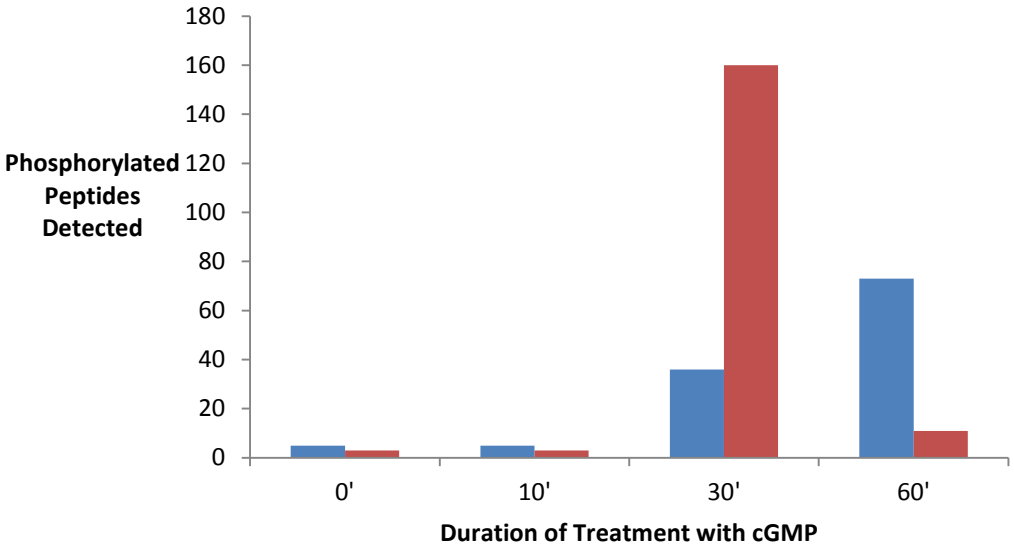

Figure 1: Comparison of identified phosphorylated proteins in the present study and the P3DB database. The number of proteins detected as being phosphorylated by MS that were already registered as phosphorylated in the P3DB database are represented in blue bars, while the novel phosphoproteins detected are represented in red bars.

Figure 2 shows increasing numbers of unique proteins detected at each time point among the enriched samples. This correlates with the proportionally increasing detection of phosphorylated peptides. However, the number of unique detected proteins is significantly higher than the number of phosphorylated peptides detected. The unenriched samples also have increased numbers of unique proteins detected over time, aside from the very high reading in the 30 min unenriched sample, but the increase is less dramatic than the escalation observed in the enriched samples.

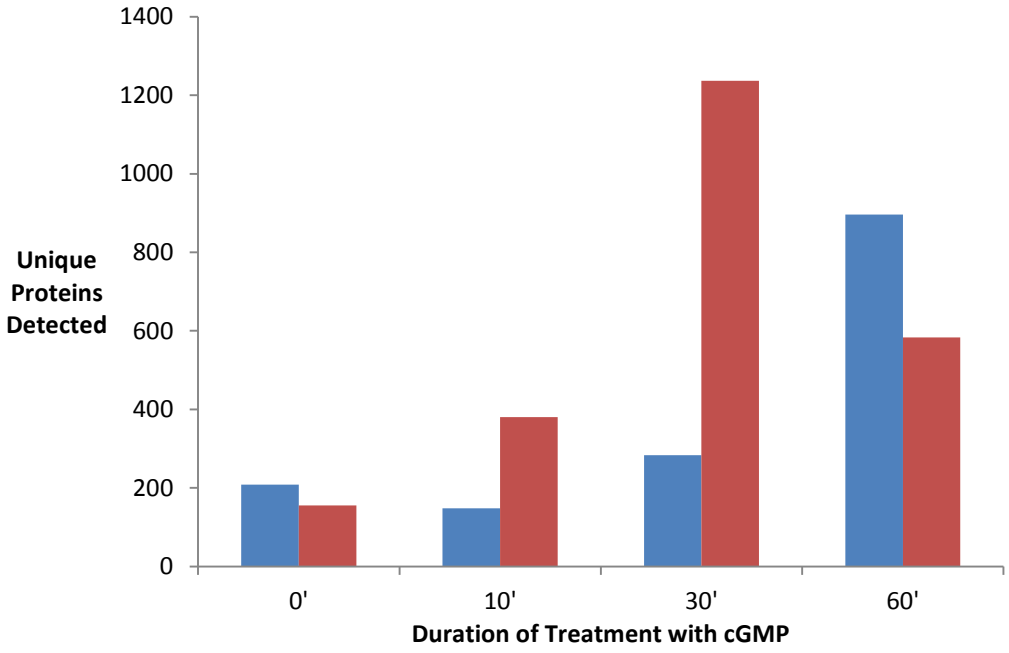

Figure 2: Unique proteins identified at each time point in enriched and unenriched samples. This figure shows the abundance of unique proteins detected in both the enriched (blue) and unenriched (red) protein samples that were analyzed using MS.

3.3 DISCUSSION

It is clear that cGMP treatment affects the phosphorylation of many proteins, and likely does so in a long time frame. In the natural system, cGMP levels usually build over multiple hours in response to stimuli [10] as the molecule serves as a second-messenger in the cellular signaling pathways. Therefore, though it is reasonable that cGMP has a long-term effect, it is somewhat surprising that cGMP has a continuously growing effect on phosphorylation over a long period when introduced and maintained at a high level. This demonstrates that cGMP's effect on

phosphorylation signaling is complex due to the demonstrated time-delayed effects. Although it is possible that a cascade of phosphorylation events is occurring over an extended period of time, this is not the traditional timeframe that is expected for a phosphorylation cascade [9]. Our results indicate that new proteins are being phosphorylated between each time point. This suggests that translation of new proteins may be occurring as a result of the cGMP treatment and that these proteins are then phosphorylated.

A comparison between unique proteins identified in unenriched and phosphopeptide enriched protein samples showed that the 30 min time point sample had the highest number of proteins identified in the unenriched sample (**Figure 2**). This large number of detections correlates to the increased detection of phosphoproteins at the same time point (**Figure 1**). Of interest, the 30 min time point has a very high rate of phosphorylated peptide detection corresponding to a similar abundance of proteins that were not in the database. This data needs further clarification to determine the accuracy of these phosphorylation sites, as the simplest explanation for this observation is that there were many false positives in this sample. In order to ascertain whether the high rate of phosphorylated proteins at this time point is an accurate reading, at least two replicates will be analyzed and those peptides present consistently in at least two of the replicates will be used to verify this claim.

One interesting trend is that the number of proteins detected in the enriched sample generally increases over time. Assuming a more nominal value for

the 30 min unenriched sample, the same trend of increasing detection exists in the unenriched samples, although it is not as drastic. We would expect that the overall content of the total proteome would not change much, and so this increase in the unenriched samples is suspect. Therefore, despite the trend revealed in these preliminary results, it is important to perform a similar analysis on at least two replicates as a means of attaining statistically confident analysis. The increase described could be due to a dramatic increase in the translation of new proteins during this time, but may also be an experimental anomaly. If it is an experimental anomaly, it gives rise to additional uncertainty in the increasing phosphorylation trend seen in the enriched samples. The analysis of the remaining two replicates should allow for statistically supported results by providing the ability to account for noise in the signal and dismiss or further examine measurements that appear anomalous at the moment.

4. METHIONINE OXIDATION

4.1 INTRODUCTION

4.1.1 Methionine Oxidation Believed to be Primarily Damage

Methionine oxidation has been the subject of research as early as the 1950s when it was suggested that reduction of methionine sulfoxide could be catalyzed by an *E. coli* enzyme [11]. At the time, the effect of methionine oxidation was unclear, but it was assumed to be just cellular damage [12, 13]. It was not until 1981 that a team working with *E. coli* was able to partially purify the first Methionine Sulfoxide Reductase (MSR), which is an enzyme capable of reducing methionine sulfoxide to unmodified methionine [12] (**Figure 3**). At that time, the protein was assumed to act solely as a repair enzyme to reverse oxidation damage. This assumption has been preeminent throughout the literature, with many reports researching medical implications of methionine oxidation e.g. in late-onset diseases such as Alzheimers and Parkinsons [14-16]. As support that methionine oxidation may contribute to accumulative oxidative damage, it has been noted that mammals lack the enzyme necessary to reduce free methionine sulfoxide residues of the R-enantiomer [17]. This view has been supported by literature describing the increase of methionine sulfoxide that is implicated in both aging and stress in multiple species [3, 18].

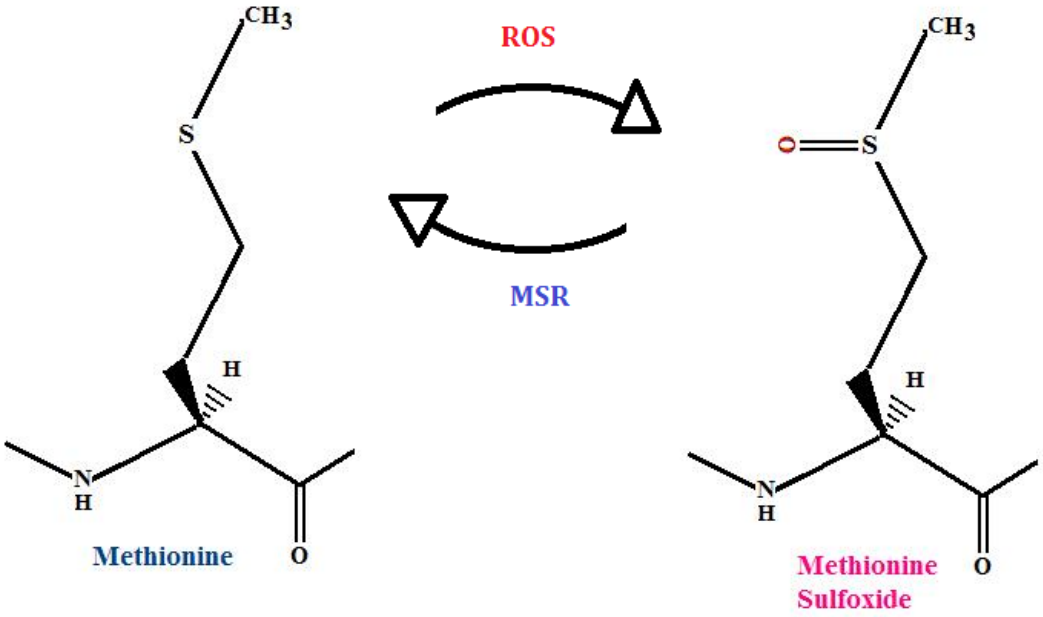

Figure 3: Methionine oxidation and reduction cycle.

The significance of MSR in the prevention of oxidative damage to the cell has been explained in two progressively more complex theories. In both theories, oxidation is assumed to be solely an undesirable cellular damage. The first hypothesis is supported by experimental data that indicates that oxidized methionine residues occurring close to phosphorylation sites can prevent the phosphorylation and thereby affect the functionality of the pathway [19]. It was thus proposed that MSR is required for repair of the damaged proteins and hence the functional restoration of the pathway [20]. In this theory, methionine oxidation is linked to signaling, but only in the sense that its effects can render signaling pathways dysfunctional. An additional theory has been postulated suggesting that MSR might actually serve to scavenge excess reactive oxygen species (ROS) in the cellular environment [21, 22]. Methionine residues are the most easily oxidized by

ROS, and thus protect more sensitive residues from the damaging effects of oxidation. The MSR is then able to restore these methionine sulfoxide residues to methionine using reductive potential, generally in the form of thioredoxin. This second theory suggests that MSR is a critical repair enzyme that is responsible for protecting the cell from oxidative damage.

4.1.2 Methionine Oxidation as a Cellular Signal

Recent work on methionine oxidation has demonstrated that it is also capable of regulating redox homeostasis. One of the best studied mechanisms of how such a regulation acts is via calmodulin [23, 24]. The calmodulin molecule has nine methionine residues that can be oxidized resulting in altered functionality. This altered functionality can then serve as a feedback loop to inhibit the generation of ROS and maintain redox homeostasis in plants [23, 24] by effecting a change in the level of cellular respiration. As the cell environment becomes more oxidized, methionine residues on calmodulin are oxidized, which effects a reduction in cellular respiration and a decrease in the generation of ROS. Although this feedback loop appears to be rather simple, it demonstrates that methionine oxidation can be harnessed as an adaptive signaling mechanism (**Figure 4**).

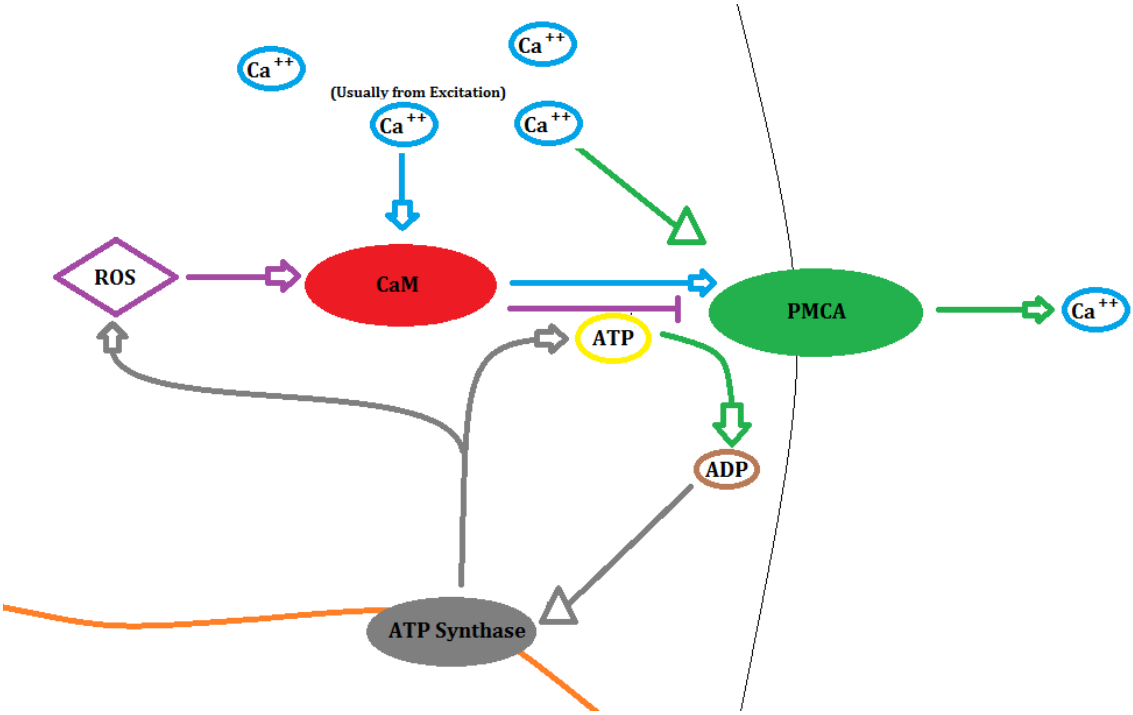

Figure 4: The effect of ROS on the function of CaM. When ROS oxidizes a methionine on CaM, there is not activation of the Plasma Membrane Calcium-ATPase (PMCA) ion pump, which leads to a decrease in ATP demand. High levels of ATP slow oxidative phosphorylation processes and thus reduce the generation of ROS.

Additional studies have looked at other ways in which methionine oxidation can affect the function of proteins other than calmodulin, specifically those involved in phosphorylation- or calcium-dependent signal transduction, and it has been demonstrated that the ability to phosphorylate substrates by both plant calcium-dependent kinases and human AMP-dependent protein kinase can be decreased by oxidation of key methionine residues in the substrate [19]. These methionine residues seem to serve as recognition sites according to the study. When they are oxidized, they become more hydrophobic [25] which may prevent kinases from binding properly. It was demonstrated [19] in both human and plant kinases that

oxidation of key methionine residues in known substrates prevented kinase recognition and activity. Again, this was originally understood to be simply the result of damage, but it has since been suggested to be a signaling step in its own right. In 2000 it was claimed that H_2O_2 meets the physical conditions necessary of an intracellular messenger, such as diffusibility and degradability, as well as an externally inducible response [26]. Examples of ligands that are known to induce an H_2O_2 response include epidermal growth factor, insulin, platelet derived growth factor and tumor necrosis factor alpha [26]. An experiment in which catalase was added to rat smooth muscle cells treated with platelet derived growth factor demonstrated that H_2O_2 was necessary for the MAPK activation that would occur in the absence of catalase [27]. This suggested that proper signal transduction likely includes the oxidation of key residues in response to H_2O_2 production or detection.

Despite the evidence indicating that H_2O_2 appears to be a necessary signal in at least some pathways, coupled with the favorable physical characteristics of diffusibility and degradability, doubts remained because H_2O_2 and other ROS may be considered to be too small and simple to have sequence specific interactions with proteins [26]. Still, it was noted [26] that some methionine residues that are more likely to be oxidized based upon the characteristics of the residues surrounding them, indicating that there might be specificity. The specificity is thought to be based on increased reactivity caused by a lowered pKa in methionine residues surrounded by polar groups, making the oxidation of the residue occur more easily. Additionally, a recent review describing human cell responses proposes that MSR

might have substrate specificity [1], thereby reducing some oxidized methionine residues more quickly than others. Furthermore the authors claim that this specificity is likely significant in determining the oxidation patterns within the cell. Taken together, this information suggests that ROS could actually serve as true signaling molecules that are capable of targeted signal transduction by acting with reactive specificity rather than conformational specificity.

4.1.3 Methionine Oxidation as Part of a Complex Signaling System

A review published in 2006 [2] discussed ways in which high-throughput mass spectrometry-based protein analyses can be used to better understand post-translational oxidative modifications of proteins. The report also presents evidence that strongly indicates that oxidation is related to signaling rather than just damage [2] by describing how amino acid oxidation exhibits both specificity and reversibility and presents a table of 25 different oxidation events that were shown to affect the behavior of the protein in a dramatic way, including both deactivation as well as activation. As an example, it was determined that the cAMP-Responsive Element Binding (CREB) transcription factor of humans experiences enhanced DNA binding when two of its cysteine residues are reduced [28]. Most of the listings are related to cysteine oxidation, which has been established as a signaling pathway for some time and has thus been more thoroughly explored. However, more recent work on methionine has led to the publication of additional methionine oxidation events with suggested signaling roles, in *E. coli,* as well as both plants and human cells [19, 23, 24]. It seems likely that further pathways utilizing methionine

oxidation will be described as methionine oxidation is accepted as a possible method of signal transduction.

In addition to the large number of individual protein studies demonstrating the dramatic effects that can occur in response to oxidation, there are also some key studies that hint at the scope and significance of signaling related to methionine oxidation. One of the most compelling reasons that the hypothesis of oxidative signaling has been questioned is the low conformational specificity of ROS in comparison to more established signaling pathways, such as those that include kinases. In addition to the specificity of oxidation that is determined by the pKa of residues, a report published in 2011 describes how the critique of a lack of specificity may be even less true of methionine oxidation because of the role of MSR [29]. As discussed above, the MSR family is part of an enzyme family that demonstrates some specificity in reducing methionine sulfoxide to methionine. However, it accomplishes this reaction as a catalyst, simply allowing the reduction event to occur where activation energy barriers between the reducing agent and the oxidized methionine would otherwise prevent it. It was therefore suggested that it might actually be possible that some MSR enzymes could be serving as oxidase enzymes under specific cellular conditions that were more highly oxidizing. This would allow a sudden release of ROS to potentially have a catalytically-directed effect on specific proteins, which would constitute a *bona fide* signaling mechanism.

In transgenic *Arabidopsis thaliana* plants improved stress tolerance was obtained when a *Zea mays* transcription factor was constitutively expressed [30].

When the ABA-responsive transcription factor ABP9 is constitutively expressed in *A. thaliana*, cellular levels of ROS are markedly decreased, corresponding to an increase in expression of many stress-related genes in the plants. These trends also correlated to a decrease in cell death. It was therefore argued that, while oxidation in cells can be damaging at high levels, it may also serve as a signal at moderate levels and that the transcription factor may serve to more tightly regulate the stress response [30]. In light of the importance of oxidation in signaling, it seems quite possible that the changed levels of ROS are directly linked to the improved stress response as damagingly high levels of oxidation may be regulated down to appropriate signaling levels via some action of the *Zea mays* transcription factor.

4.1.4 Experimental Overlap with Phosphorylation

Due to the emerging role of methionine oxidation in signaling, the same samples from the phosphorylation analysis were used to screen for proteins with oxidized methionine residues. Though methionine oxidation has not previously been a focus in our lab, this was made quite effective as a paired strategy, because enrichment for phosphoproteins also enriched our samples in oxidized proteins due to the negative charge of both groups. Therefore, data on both modifications was obtained simply by changing variable modification on MASCOT search using the same MS spectra.

4.2 RESULTS

The analysis of methionine oxidation took two forms; first, it included the bulk analysis of methionine oxidation trends in response to cGMP, secondly, the specific proteins reacting to cGMP were identified and analyzed using data from the TAIR website [7]. Despite the lack of an ideal number of repetitions, the methionine oxidation response was still statistically significant due to the very strong response that was detected. A very low level of methionine oxidation was detected in the control (untreated sample) but the level of methionine oxidation increased dramatically in the treated 10 min sample and remained high through the 60 min sample as can be seen in Figure 4. This suggests a very strong oxidation of methionine in response to cGMP treatment. The error bars are quite large in the figure, due to a lack of replicates available, but it can be seen that the control is quite clearly outside the standard deviation of the set.

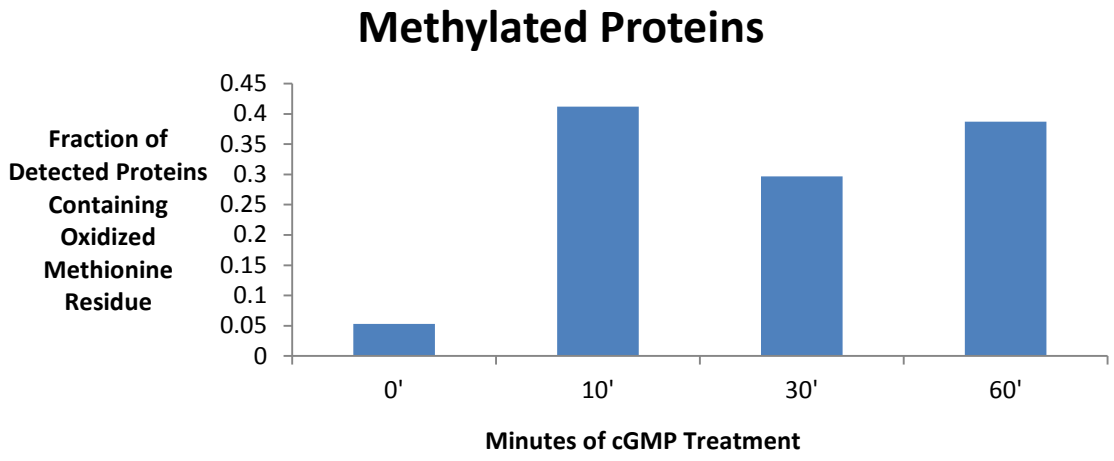

Figure 5: Proportion of enriched proteins containing oxidized methionine residues. Samples of *Arabidopsis thaliana* suspension culture cells were treated with cGMP for various times. Proteins were then extracted and enriched using TiO_2 bead columns and then analyzed using MS.

A closer look at the individual genes from the cGMP treatment of *Arabidopsis thaliana* cell suspension culture reveals 8 proteins that were detected throughout the experiment and also exhibit *de novo* oxidation in response to cGMP. Interestingly, of these 8 proteins, 3 of them are known to be responsive to cold stress, including both CCR1 and CCR2 (Cold Circadian Rhythm and RNA Binding Proteins 1 and 2).

4.3 DISCUSSION

In agreement with the emerging body of evidence suggesting that methionine oxidation is a signaling mechanism [1, 19, 23], our data suggests that methionine oxidation likely plays a role in signaling in response to cGMP. Though it will be more certain when further replicates are processed, the data already yields the statistically significant result that methionine oxidation increases in response to cGMP treatment. This is a very important result, because cGMP represents a native signaling molecule that, by itself, could not oxidize any residues. Therefore, such an increase in oxidation must be the result of some responsive biological process. Though this process could be a damage-related process, the evidence of methionine oxidation-responsive pathways, such as the example of calmodulin's control of redox state through the methionine oxidation signal [23, 24], suggests that this may

be an example of methionine oxidation occurring to modify cellular behavior and signaling pathways.

The three cold-related proteins that become oxidized in response to cGMP treatment provide an interesting target for further research. The protein CCR2 is known to oscillate through a negative-feedback loop with itself, and expression can be induced by cold [7]. CCR2 causes stomatal opening during salt or drought stress, thereby reducing tolerance, but induces stomatal closing during cold stress, improving cold tolerance. Loss of function mutants also have an increased susceptibility to pathogens, indicating that it may play a role in host defense [7]. Due to the fact that CCR2 seems to have the ability to either benefit or harm the plant, it is an example of a protein that must be carefully controlled in order to maintain optimal fitness. These proteins are especially interesting due to the fact that they are harmful under conditions of drought, while they are beneficial under conditions of cold stress [7]. In conclusion, it is suggested that methionine oxidation might be a means of preventing undesirable activation of these proteins under the conditions which would make them harmful.

5. CONCLUSION

Mass spectrometry is an invaluable tool in surveying the proteome and post-translational modifications. While mass spectrometry has allowed for the analysis of single proteins via isolation of proteins separated using 2-D gel technology for some time, the software and hardware required for whole proteome analysis has and will continue to have a dramatic effect on proteomics research. The data presented in this thesis is a result of this technological development, and exists as perhaps the first examination of the entire proteome in response to cGMP treatment.

Since cGMP's signaling pathways are poorly understood at present [5], the expectation is that many of the signals may be passed through post-translational modifications that have not been able to be analyzed using transcription-level detection methods. With the development of MS technologies that are sensitive enough to identify post-translational modifications in high-throughput experiments, it is now possible to explore the potential signaling pathways of cGMP. This experiment aimed to examine phosphorylation and methionine oxidation for any potential in cGMP-induced signaling response.

In regards to phosphorylation of proteins in response to cGMP, the data available at this point reflects the lack of sufficient replicates in its low quality. However, with that in mind, there is still valuable data to be extracted from the experiment. Most importantly, this experiment demonstrates that there is an increase in phosphorylation of proteins in response to cGMP treatment. Additionally, the time frame of the response is longer than might have been

assumed given the steady and high concentration of cGMP used to treat the cells. The data shows that the degree of protein phosphorylation increases throughout the time frame of the experiment, suggesting that cGMP elicits a response that is more than just immediate. Though cGMP normally acts over a multiple-hour time frame due to slowly increasing levels [10], it might have been expected that a higher level of cGMP would elicit all effects in a shorter time frame and then remain relatively stable in the long term. It would not have been unreasonable even to expect decreasing after some time as negative feedback pathways returned the cell to a basal state. However, with the number of phosphorylated proteins continuing to increase past the final 60 minute time point, it seems likely that cGMP plays a role in long-term signaling even when introduced at a high level. This will become clearer following a more careful exploration of the proteins involved and the repeatability of their response in order to determine whether this phosphorylation response is part of a controlled, repeatable signal or uncontrolled damage.

Unlike the phosphorylation response, the response of increased methionine oxidation is statistically significant without performing additional replications of the experiment. However, like the phosphorylation data, the clarity of the methionine oxidation response is dependent upon further sample analysis. The data strongly indicates that methionine oxidation is occurring in response to cGMP and, coupled with both the large body of research supporting methionine oxidation as a signaling mechanism and the brief analysis of the proteins which were detected to become oxidized, this response suggests that methionine oxidation may be part of the cGMP

signaling pathway. It has been suggested that cGMP signaling requires a signaling mechanism that is not “on/off” in function [10], and methionine oxidation may be this signal. Unlike the phosphorylation response of the proteins, which showed an increase in the number of phosphorylated proteins over time, the oxidation of methionine showed only a statistical difference between the control and the treated samples. Though it is possible that the trend is positively correlated with increasing time, the data is not clear enough to suggest a statistically significant trend. If methionine oxidation is occurring relatively rapidly in response to cGMP treatment, it is possible that it could be serving to modulate other responses including the phosphorylation response.

The three genes related to cold stress that were detected as having oxidized methionine residues after treatment with cGMP are of particular interest, as they are likely candidates for regulation resulting due to methionine oxidation. The genes control stomatal opening, in addition to other processes, and are known to induce stomatal opening during some stresses in which it is unfavorable, as well as prevent opening in response to some stressed where closed stomata provide an advantage [7]. Since stresses often activate similar pathways, the cell would benefit greatly from having a mechanism to prevent improper activation of these proteins during the wrong stress response, and they are thus a good focus for further study. If methionine oxidation is occurring on these genes only under certain conditions related to these stresses, it would provide evidence that the methionine oxidation might be serving as this control mechanism to prevent detrimental activation of

these proteins. Additionally, exploring the functional effect of methionine oxidation on these proteins could also support or refute the hypothesis that they may be involved in a methionine oxidation signaling pathway. Together, these experiments could provide evidence that would strongly support the research on methionine oxidation as an intracellular signal.

One way to advance the research on methionine oxidation signaling, as well as proteomic studies of many other sorts, would be to establish and make publically usable a searchable database for depositing proteomic data such as that generated in this experiment. As with the generation of databases from microarray data, such databases allow for efficient use of data in order to advance science by efficiently utilizing previously generated data to explore new and emerging questions, as well as to judge the correctness of newly generated data [31-33]. As demonstrated by the value of our data in exploring both phosphorylation and methionine oxidation, proteomic data is immense and there is still much to learn from the data even after it has been used to answer the question for which it was generated.

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